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TECHNICAL MANUSCRIPT 597

# RELATIONSHIP BETWEEN PLAQUE ASSAY AND MOUSE ASSAY FOR TITRATING RIFT VALLEY FEVER VIRUS

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APRIL 1970





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# RELATIONSHIP BETWEEN PLAQUE ASSAY AND MOUSE ASSAY FOR TITRATING RIFT VALLEY FEVER VIRUS

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Process Development Division
AGENT DEVELOPMENT & ENGINEERING LABORATORIES

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April 1970

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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ABSTRACT

A plaque assay method for the small-plaque variant of the pantropic van Wyk strain of Rift Valley fever virus is described. The ease in preparation, the reproducibility of results, the distinctness of the plaques, and the relatively short time required for plaques to appear make the method highly suitable for use in the laboratory. A model was constructed utilizing this technique for estimating $MICLD_{50}$ values from plaque-forming units. The model obtained has provided a firm basis for a large reduction in costs of routine assays, due to its relative costs of mouse and tissue culture assay.

## I. INTRODUCTION*

A number of investigators who successfully plaqued Rift Valley fever virus (RVFV)[1-6] observed plaques 1 mm in diameter; Runnels and Brown[7] later described large plaques up to 5 mm in diameter. Undoubtedly, the above-mentioned plaques were all of the wild pantropic virus, which suggested variants in wild pantropic RVFV. It was not until recently that Doyle[8] plaqued the wild pantropic strain of RVFV and found plaques of various sizes either before or after passage in mouse fibroblast tissue cell cultures. He was able to select and characterize the small-plaque and large-plaque variants of the pantropic strain of RVFV. He further demonstrated that the small-plaque variant was more virulent for mice than the large-plaque variant. The present report describes the relationship between the in vivo infectivity using mice and in vitro plaque development in an L cell clone. The criteria of linearity and reproducibility were met and proven. A model is also presented for estimating the mouse intracerebral lethal dose$_{50}$ (MICLD$_{50}$) from plaque-forming units (pfu). The model obtained has provided a firm basis for a large reduction in costs of routine assays for RVFV in these laboratories, due to the relative costs of mouse and tissue culture assay. The authors recommend a similar study to determine if a comparable relationship holds for any virus assay system routinely performed.

## II. MATERIALS AND METHODS

### A. VIRUS

The small-plaque variant of the pantropic van Wyk strain of RVFV[8] was used in this study. The origin and maintenance of this strain were described by Walker et al.[9]

Virus was propagated in L-DR strain, a variant of Earle's L cell that was grown in medium supplemented with bovine serum.[9] Multiplicity of inoculum (MOI) was 0.01, cell concentration was 2 x $10^5$ cells/ml, and the infection period was 48 hours. The tissue cells were removed by low-speed centrifugation and the virus was assayed.

### B. CELL CULTURES FOR PLAQUING

A variant (L-MA) of Earle's L cell was obtained from Dr. Donald J. Merchant, University of Michigan, Ann Arbor, Michigan. The cells were maintained antibiotic-free, and a selected clonal line designated L-MA clone 1-1 was established as the tissue cell line in these studies. Growth medium for L-MA clone 1-1 was 199 medium supplemented with 0.5%

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Bacto-Peptone. Cultures were grown in suspension culture[10] to produce bulk quantities that were determined to be PPLO-free by plating samples on Mycoplasma agar.* Frozen stocks (-175 C) were prepared and used routinely to avoid inconsistencies in plaque efficiency due to continuous cell passage.

## C. PLAQUE OVERLAY MEDIUM

The plaquing medium was composed of two overlays. The first overlay medium consisted of two parts: (i) 1X Hanks BSS with 1.0% Noble agar, which was autoclaved at 10 psi for 10 min, and (ii) 20% calf serum plus 69% 199 peptone medium containing a 1.0% solution of sodium bicarbonate. Equal volumes of both component parts were individually warmed to 50 C and then mixed; 1% Pen-Strep, 1% Mycostatin, and 1% (100 μg/ml) DEAE-dextran were then added.

The second overlay medium also consisted of two parts: (i) 1X Hanks BSS with 1.0% Noble agar and (ii) 77% 199 peptone medium containing a 0.5% solution of sodium bicarbonate. Equal volumes of both components were individually warmed to 50 C and then mixed, with the addition of 0.007% of a neutral red solution, 1.0% Pen-Strep, and 1.0% (100 μg/ml) DEAE-dextran.

## D. PLAQUE ASSAY

Monolayers were prepared by seeding 2-ounce bottles** with 5 ml of cell suspension at $7.5 \times 10^5$ cells/ml in growth medium. After incubation overnight at 37 C, the growth medium was removed and cells were washed with 1X Hanks BSS at pH 7.6. Each bottle was inoculated with 0.1 ml of virus diluted to the desired concentration and allowed to adsorb for 1 hour at 30 C. After adsorption, 5 ml of the first overlay medium at 45 C were added to each bottle. When the overlay had solidified (1 hour), the bottles were inverted and incubated at 37 C for 72 hours. The monolayers were then removed from incubation, and the second overlay, which contained stain for plaque observation, was applied. Plaque preparations were covered with heavy brown paper from time of inoculation until addition of second overlay to avoid photoinactivation. The plaques were counted at 1 hour and again at 24 hours. All dilutions were counted, and the pfu values were calculated from dilutions having the highest number of plaques. In almost all cases, counts were made on bottles containing 50 to 150 plaques.

For reisolation of the virus, plaques were removed by an inoculating loop of heavy-gauge wire. Single plaques were inoculated onto confluent monolayers of L-MA clone 1-1 in 2-ounce bottles. The inoculated monolayer cultures were incubated for 24 hours at 37 C, after which the medium was removed and assayed both in mice and by the plaque technique.

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* Grand Island Biological Company, Grand Island, N.Y.
** Sani-Glass Flint-Grad, Brockway Glass Co., Inc., Brockway, Pa.

### E. MOUSE ASSAY

The Fort Detrick Swiss-Webster strain of mice weighing 6 to 8 g was used for titrating RVFV. Mice were inoculated intracerebrally, four mice per dilution, with 0.03 ml of viral challenge material. Deaths were recorded for 6 days postinoculation, but only those occurring after 24 hours were used in the calculation of $MICLD_{50}$. The probit method[11] of calculating $MICLD_{50}$ values was used.

## III. RESULTS

### A. PLAQUE ASSAY

Somewhat rounded, clear plaques with sharp boundaries were visible as early as 1 hour after the second overlay, which contained neutral red, was placed over the 72-hour-old culture. They were <1 to 3 mm in diameter. Statistically, there was no difference in counts taken at the 3rd and 4th day after inoculation of monolayers. The plaques were distinct, uniform in size, and easily visible with the naked eye or with an indirect lighting system if detail was desired (Fig. 1).

### B. RELATIONSHIP BETWEEN PLAQUE NUMBERS AND VIRUS CONCENTRATION

The relationship between the number of plaques detectable and the concentration of virus was determined. Starting at primary virus dilutions of $10^{-2}$, $10^{-3}$, or $10^{-4}$, successive twofold secondary dilutions were made of the different virus preparations to test for precision and linearity of the data. The plaque assay performed equally well on each of the two virus preparations (Table 1). There was excellent agreement of pfu values for all secondary dilutions and no real difference between the day 3 and day 4 pfu readings, as indicated in the last two columns. Moreover, there is a linear relationship between the average number of plaques obtained for each virus dilution and the relative concentration of virus.

### C. COMPARISON OF RVFV INFECTIVITY TITERS OBTAINED BY $MICLD_{50}$ AND PLAQUE ASSAY

In preliminary experiments, the comparison of RVFV infectivity titers between pfu and $MICLD_{50}$ was 1.5 logs higher for $MICLD_{50}$ (Table 2). This difference was based on titrations of cultures all approximating the same degree of infectivity. In most cases, this meant that $MICLD_{50}$ values were higher than pfu titers, suggesting that the $MICLD_{50}$ was somewhat more sensitive (approximately 31.5 $MICLD_{50}$/pfu).

FIGURE 1. Plaques of Rift Valley Fever Virus in L-MA Clone 1-1 Tissue Cells 72 Hours After Inoculation of Virus.

TABLE 1. RELATIONSHIP BETWEEN PLAQUE COUNT AND VIRUS CONCENTRATION

| Virus Preparation and Concentration | Dilution | Plaques/Bottle | | $Log_{10}$ pfu titer | |
|---|---|---|---|---|---|
| | | Number | Average | Day 3 | Day 4 |
| 48-Hour Infectious Culture | | | | | |
| x $10^{-3}$ [a] | 1 | 239, 242 | 240.5 | 6.38 | 6.38 |
| | 1/2 | 125, 120 | 122.5 | 6.39 | 6.37 |
| | 1/4 | 63, 63 | 63.0 | 6.40 | 6.38 |
| | 1/8 | 27, 20 | 23.5 | 6.27 | 6.32 |
| x $10^{-4}$ | 1 | 68, 79 | 73.5 | 6.87 | 6.93 |
| | 1/2 | 25, 40 | 32.5 | 6.81 | 6.86 |
| | 1/4 | 9, 16 | 12.5 | 6.70 | 6.68 |
| | 1/8 | 1, 4 | 2.5 | 6.30 | 6.65 |
| Alum-Precipitated | | | | | |
| x $10^{-2}$ | 1 | 246, 232 | 239.0 | 5.38 | 5.37 |
| | 1/2 | 142, 162 | 152.0 | 5.48 | 5.52 |
| | 1/4 | 72, 87 | 79.5 | 5.50 | 5.54 |
| | 1/8 | 43, 41 | 42.0 | 5.53 | 5.61 |
| x $10^{-2}$ | 1 | 71, 32 | 52.5 | 4.71 | 4.74 |
| | 1/2 | 25, 14 | 19.5 | 4.59 | 4.66 |
| | 1/4 | 10, 21 | 15.5 | 4.79 | 4.82 |
| | 1/8 | 4, 3 | 3.5 | 4.45 | 4.45 |
| x $10^{-3}$ | 1 | 255, 247 | 251.0 | 6.40 | 6.40 |
| | 1/2 | 175, 165 | 170.0 | 6.53 | 6.53 |
| | 1/4 | 104, 105 | 104.5 | 6.62 | 6.68 |
| | 1/8 | 58, 79 | 68.5 | 6.74 | 6.80 |
| x $10^{-3}$ | 1 | TNTC, TNTC[b] | - | - | - |
| | 1/2 | 217, C[c] | 217.0 | 6.34 | 6.35 |
| | 1/4 | 96, 101 | 98.5 | 6.60 | 6.61 |
| | 1/8 | 49, 60 | 54.5 | 6.64 | 6.71 |

a. Each dilution studied was a separate virus pool that differed in treatments.
b. Too numerous to count.
c. Contaminated.

TABLE 2. COMPARISON OF MOUSE AND PLAQUE ASSAY OF RIFT VALLEY FEVER VIRUS

| Assay Procedure | Number of Replications | Mean $Log_{10}$ Titer | Standard Deviation | Variance[a/] $s^2$ |
|---|---|---|---|---|
| $MICLD_{50}$ | 42 | 8.2 | 0.30 | 0.900 |
| Plaque | 42 | 6.7 | 0.39 | 1.522 |

a. Significant at the 6% level.

To further test the relationship and possible difference in linearity between the two assay systems, the second in these series of experiments was performed. After low-speed centrifugation, the supernatant was: (i) stored at -65 C or (ii) alum-precipitated and stored at -65 C. Then, in order to determine if various virus treatments affected the titers obtained by the two assay systems, samples of each of two stored test materials were selected at random once a week for 3 weeks and assayed at five different dose levels. To obtain the dose levels necessary for showing linearity, we diluted the original samples (titer) of each of the virus cultures. The dilution levels of these samples were from $10^{-1}$ through $10^{-5}$ log, which gave five dose levels. Subsequent dilutions were made to adjust the number of plaques per bottle. Therefore, $10^{-1}$ log dilution might have five subsequent dilutions, whereas $10^{-5}$ original log dilution might have only one or no subsequent dilutions. The means for the log pfu and log $MICLD_{50}$ values were analyzed by linear regression to obtain calculated slopes and the correlation coefficients for the two assay techniques.

For this assay to be useful, a straight-line relationship has to exist between dilution and titer (virus concentration) over a wide dose range. Such a relationship was demonstrated with this plaque assay (Fig. 2). There was no statistical difference between slopes over the treatments tested. Moreover, when the same treatment material was assayed in mice, no statistical change in slopes was demonstrated. In fact, there was no statistical difference between the slopes of the plaque and mouse assay systems; however, the titer of the $MICLD_{50}$ was 0.7 to 1.0 log greater than the pfu. Examination of the data does indicate that the variability of the points about the lines of $MICLD_{50}$ is somewhat more than that of the pfu, although the difference in variability is not statistically significant.

A straight-line relationship was also obtained when the pfu values were plotted against $MICLD_{50}$ values (Fig. 3). Again, no statistical difference in slope was demonstrated over the treatments when the pfu were plotted against the $MICLD_{50}$.

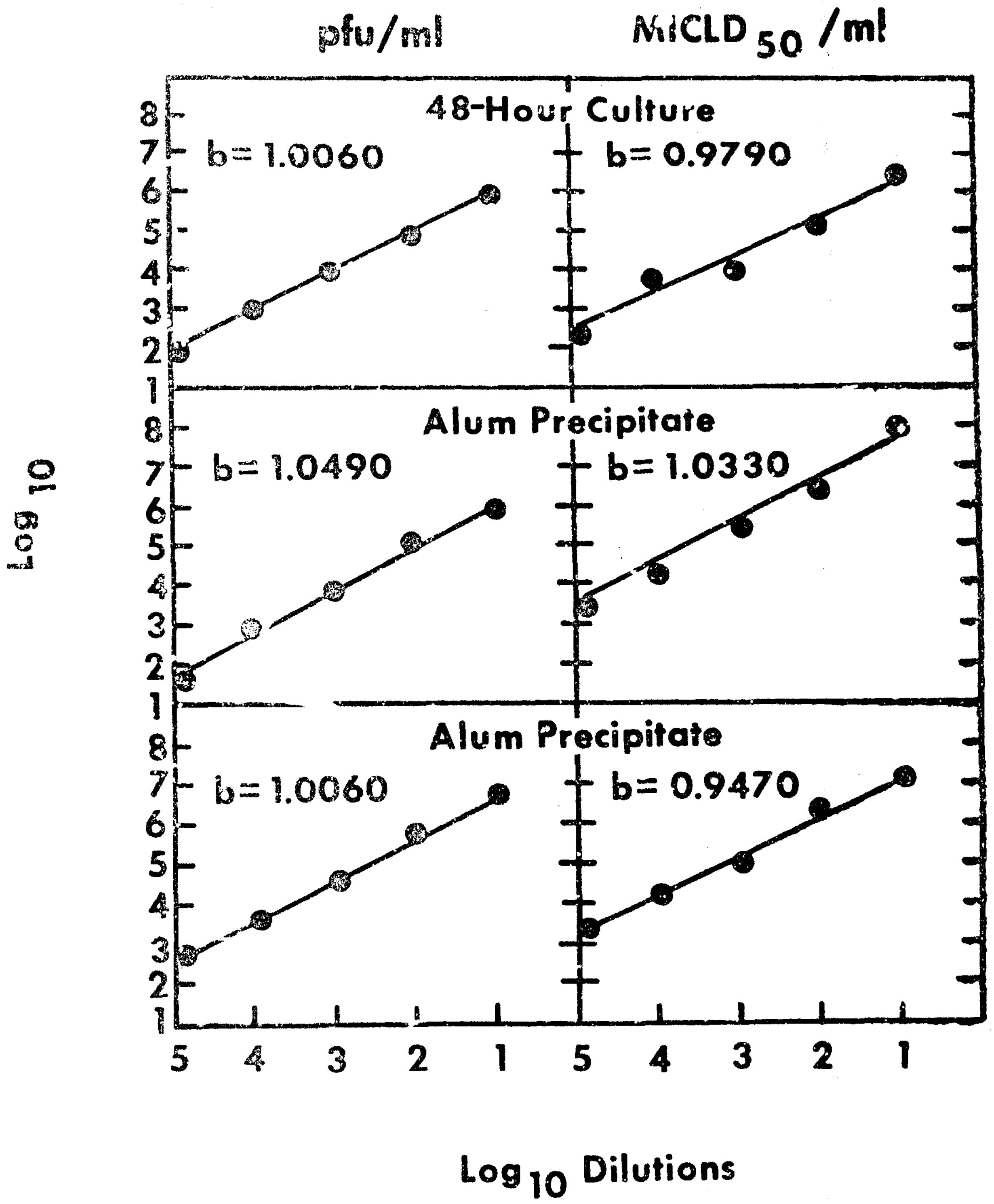


FIGURE 2. Slope Comparison Between Rift Valley Fever Virus Assay Techniques After Alum Treatment.

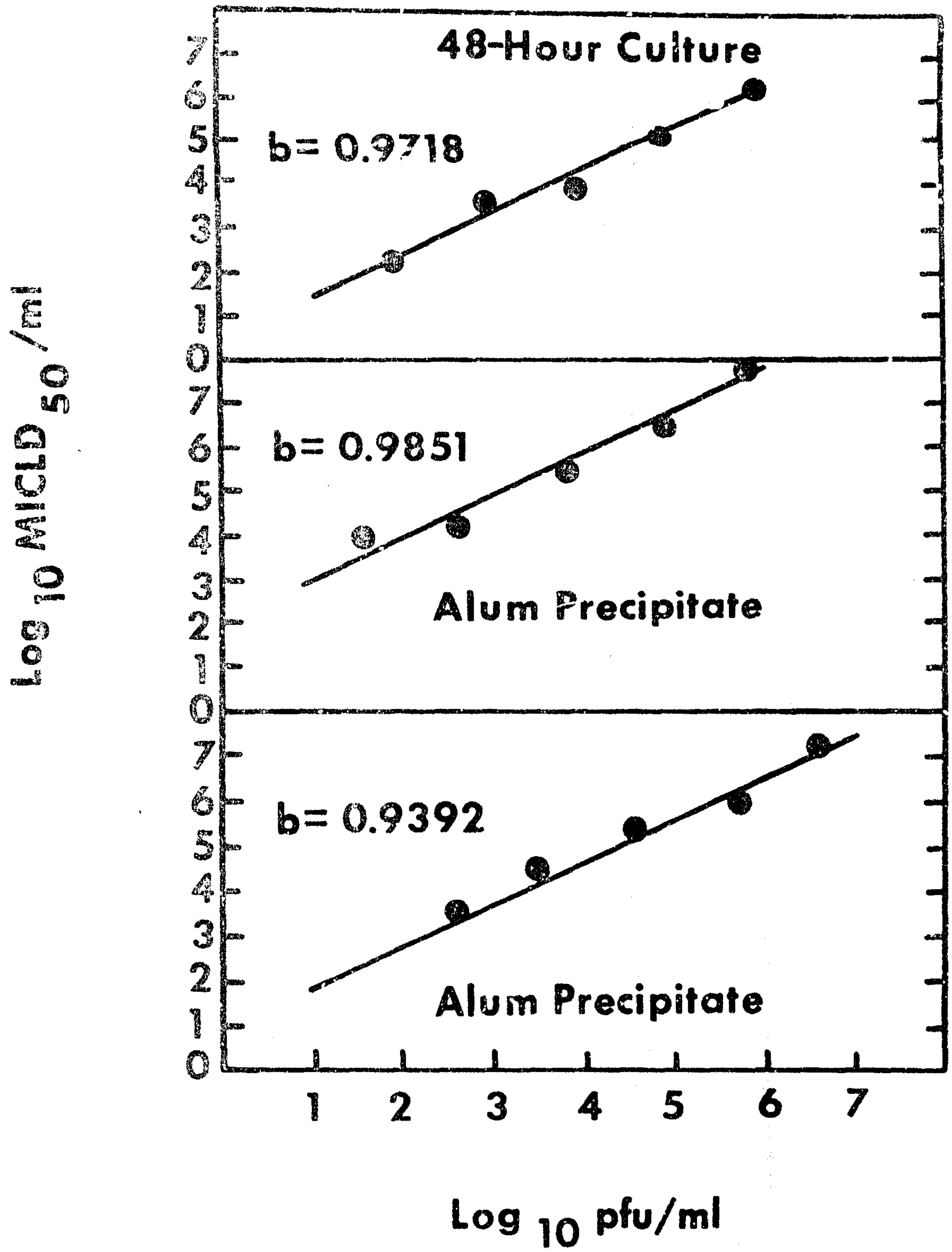


FIGURE 3. Relationship of pfu to $MICLD_{50}$ After Alum Treatment.

From this work, we concluded that the alum treatments of the virus suspensions had no appreciable effect on the plaque assay system. Therefore, all data that had been collected in our laboratory for comparing pfu and $MICLD_{50}$ could be put into one linear regression program for obtaining an estimated slope that can be used for estimating $MICLD_{50}$ from pfu (Fig. 4).

The straight line shows the estimated relationship between pfu and $MICLD_{50}$; the 95% confidence limits are represented by the inner pair of curved lines, and the 95% tolerance limits for a point that could be determined by a single plaque assay and a single mouse assay are represented by the outer pair of curved lines.

If one uses the model to estimate the $MICLD_{50}$ infectivity (potency) of test material for which the plaque assay gives 6.5 $\log_{10}$ pfu, the estimate will be 7.1 with 95% tolerance limits of approximately 6.0 to 8.3 $\log_{10}$ $MICLD_{50}$. Obviously, the present slope is restricted to 8.5 $\log_{10}$ pfu, thus restricting the range in which $MICLD_{50}$ can be estimated unless the line is extrapolated to account for higher pfu values.

D. REISOLATION OF VIRUS

RVFV was readily reisolated from plaques; a single plaque of the small-plaque RVFV inoculated into monolayer produced $10^{-3}$ to $10^{-5}$ tissue culture infectious doses per plaque. No plaques were seen in the absence of virus inocula, nor was virus recovered from clear areas of the infected agar surface. The reisolated virus was neutralized by specific hyperimmune sheep serum.

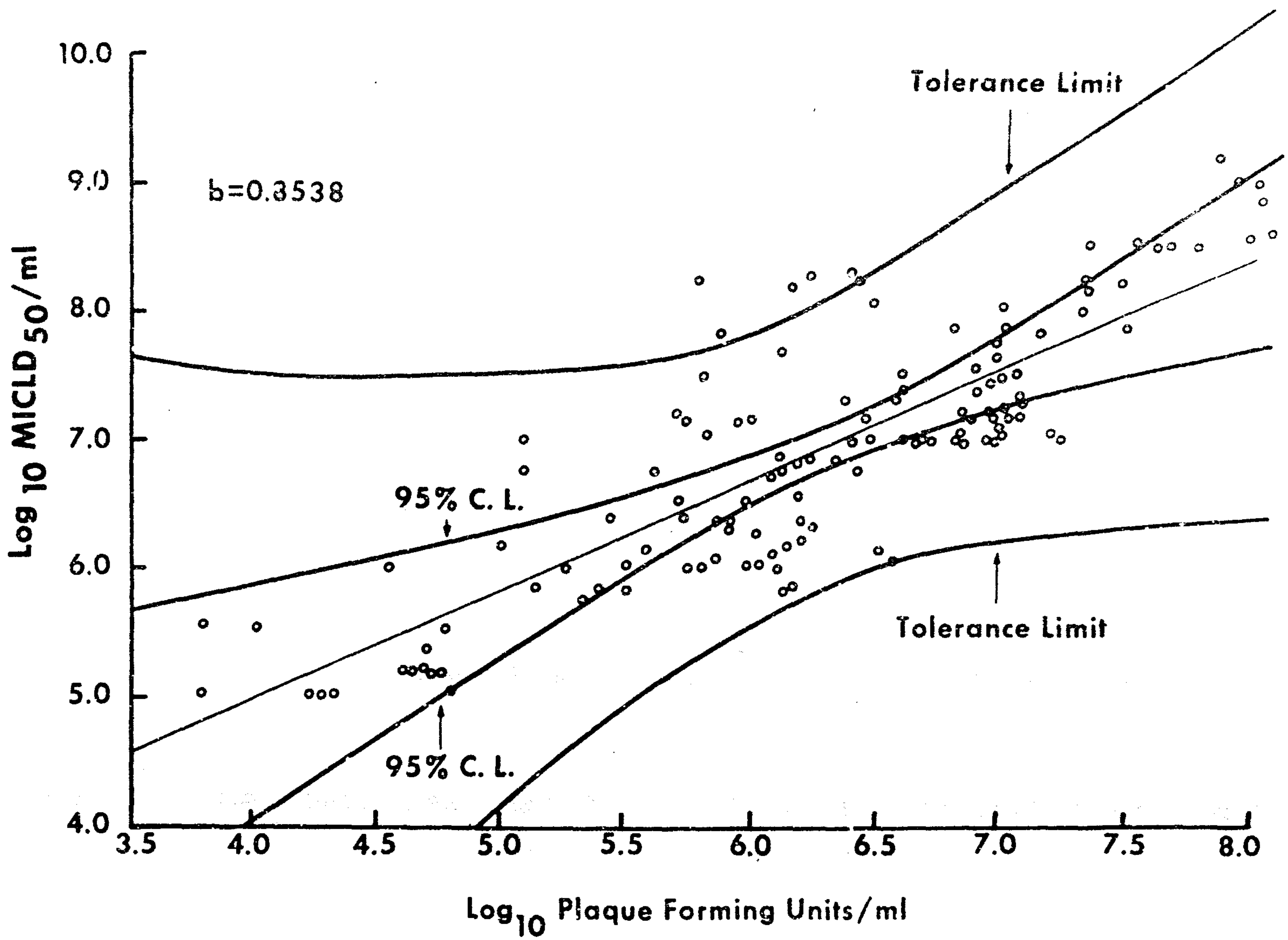


FIGURE 4. Model for Estimating $MICLD_{50}$. The straight line shows the estimated relationship between pfu and $MICLD_{50}$; the 95% confidence limits are represented by the inner pair of curved lines, and the 95% tolerance limits for a point that could be determined by a single plaque assay and a single mouse assay are represented by the outer pair of curved lines.

## IV. DISCUSSION AND SUMMARY

RVFV produced plaques that were well-defined and characteristic of the small-plaque variant in monolayer cultures of L-MA clone 1-1 tissue cells under a double agar overlay. Plaque counts on days 3 and 4 were not statistically different, although plaque size was increased by the 4th day. A linear relationship was observed between plaque counts and virus concentration under various treatment conditions, permitting precise virus assays. These results were further extended to include comparison of slopes between $MICLD_{50}$ and pfu. Although the titers for $MICLD_{50}$ were consistently higher, there were no statistical differences in or between slopes of the two assay systems. This observation permitted accumulated data comparisons between the two assay systems to be combined into one linear regression program for development of a model for estimating $MICLD_{50}$ from pfu. The model now provides a technique for observing infectivity in two independent assay systems with certain accuracy at a greatly reduced cost to an experimenter or diagnostic and experimental laboratories, where such determinations are routine. To insure that the relationship portrayed in the model continues to hold after additional passage of the virus in tissue culture lines, it will be necessary to perform studies at regular intervals to confirm the relationship between pfu and $MICLD_{50}$. This procedure will serve to spot any changes in mouse pathogenicity, unaccompanied by decrease in pfu.

For the virus system under study, a large reduction in cost has been provided utilizing the model described. The particular model and the parameter estimates obtained, while not necessarily applicable to other virus strains, give some hope that similar comparative studies may provide a basis for like reduction in cost for routine assays of other viruses. On the basis of their own experience, the authors recommend such studies where the number of assays is appreciable.

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13. ABSTRACT
A plaque assay method for the small-plaque variant of the pantropic van Wyk strain of Rift Valley fever virus is described. The ease in preparation, the reproducibility of results, the distinctness of the plaques, and the relatively short time required for plaques to appear make the method highly suitable for use in the laboratory. A model was constructed utilizing this technique for estimating $MICLD_{50}$ values from plaque-forming units. The model obtained has provided a firm basis for a large reduction in costs of routine assays, due to its relative costs of mouse and tissue culture assay.

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